

# Reforestation accelerates soil organic carbon accumulation: Evidence from microbial biomarkers

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## ABSTRACT

Soils store more carbon (C) belowground than plants and the atmosphere combined, providing a critical ecosystem service. While previous research has shown that sustainable forest management practices can increase soil C storage by enhancing plant productivity, the role of soil microbes remains elusive. We analyzed changes in plant litter, soil C, and microbial parameters across a reforestation chronosequence—with average stand ages of ~20, 80, 120, 200 and ≥300 years—to evaluate how microbial communities mediate soil C transformation and sequestration. We observed generally consistent increases in microbial biomass (lipid biomarkers), microbial necromass (amino sugar biomarkers), and soil organic C with forest age, highlighting microbial regulation of soil C accumulation. Specifically, increases in microbial biomass preceded gains in soil C, suggesting microbial lipids are an early and sensitive indicator of ecosystem restoration. We also observed a rapid increase in microbial necromass relative to bulk soil C in forests restored for 80–200 years, likely due to accelerated microbial turnover rates. These patterns suggest high plant productivity (low litter C:N ratios) during the early and middle stages of reforestation facilitates efficient microbial growth and necromass accrual in SOC stocks. As forests age, the contribution of microbial necromass to the SOC pool declines toward background levels. Our results suggest reforestation offers a positive feedback solution that mitigates climate change by efficiently sequestering soil C belowground.

## 1. Introduction

Boreal forests, located in cold temperate zones, store approximately 18% of all the carbon (C) contained within terrestrial ecosystems (Carvalhais et al., 2014). Because these systems store large stocks of soil organic carbon (SOC) (Pan et al., 2011; Scharlemann et al., 2014), small changes in land-use or management practices can alter the balance between soil C retention and release, with implications for global climate regulation. The ability of boreal forests to store C has been reduced to some extent by disturbance, e.g. fire, logging, or conversion to pasture or cropland; in addition to reducing biodiversity and ecosystem services, forest disturbance may contribute to large losses of C if microbial conversion of SOC to CO<sub>2</sub> is accelerated (Van der Werf et al., 2009; Houghton et al., 2012).

Global demand for sustainable ecosystem development has increased the adoption of management practices that facilitate forest restoration (Lohbeck et al., 2015; Crouzeilles et al., 2016). Ecologists have monitored changes in SOC stocks during forest restoration (Jandl

et al., 2007) and typically ascribe increases in belowground C to greater plant productivity, litter deposition, and root exudation (Deng et al., 2013; Piponi et al., 2016). As a result, forest ecosystem C budgets are conceptualized as a balance between sequestration of plant-derived C and respiratory losses of soil C. Although plants are key drivers of atmospheric CO<sub>2</sub> sequestration in biomass, microorganisms may be equally critical mediators of forest C cycling (Trivedi et al., 2013). Empirical data examining microbial mediation of, and contribution to, soil C cycling across gradients of forest restoration are essential for refining best-management practices (Shao et al., 2017).

Reforestation influences soil microbial community structure (Smith et al., 2015; Zhou et al., 2017) and may change the functional role microorganisms serve in soil C cycling. In fact, it is well established that microbe-mediated turnover and recirculation of soil nutrients can promote ecosystem C sequestration (Van Der Heijden et al., 2008; Bokhorst et al., 2017). During this process, microorganisms modulate soil C storage through catabolic transformation of soil organic matter (SOM) via an *ex vivo* modification pathway (Liang et al., 2017),

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liberating organic materials and nutrients for plant uptake (Bokhorst et al., 2017). In addition to facilitating the retention of plant-derived C belowground, soil microbes may contribute directly to SOM formation through the selective retention of anabolic byproducts, especially microbial cells and residues, via an *in vivo* turnover pathway (Liang et al., 2017). These anabolic byproducts may preferentially interact with soil minerals and aggregates resulting in longer-term soil C storage (Miltner et al., 2012; Cotrufo et al., 2015).

Integrating transient snapshots with the legacy effects of microbial activity is necessary to achieve a process-based understanding of SOC dynamics. Phospholipid fatty acid analyses profile extant microbial communities that are actively regulating litter and soil C turnover (Zelles, 1999; Schimel and Schaeffer, 2012). Because amino sugars extracted from soils account for a minor percentage of living microbial biomass, they can be used as biomarkers of microbial necromass (Joergensen, 2018). As a result, amino sugar levels can be used to evaluate the extent to which microbial necromass contributes to the total SOC pool, providing critical information on the ‘chronic’ responses that drive C accumulation over time (Glaser et al., 2004; Liang et al., 2016). Examining both biomarkers simultaneously could provide unique insight on the role that soil microbes play in SOM cycling and storage (Liang et al., 2008).

Monitoring ecosystem recovery following disturbances is challenging because the same system must be observed over long time periods. Chronosequence methods (i.e. space-for-time substitutions) are the established solutions to this challenge and have been used to study forest ecosystem dynamics spanning hundreds of years (Walcker et al., 2018). Monitoring changes in SOC stocks during reforestation may improve estimates of regional C-sink capacities. Microbial indicators could also improve our understanding of how microbial communities modulate soil C levels during forest recovery. Hence, the goal of this study was to explore whether soil C storage is regulated by active microbial communities (lipid biomarkers) or microbial necromass (amino sugar biomarkers). We collected soil and litter samples from five sites spanning an ecological restoration chronosequence located in a temperate forest in the Changbai Mountain National Nature Reserve, China. We examined (1) whether SOC accumulation was positively related to the degree of reforestation; (2) how the distribution of microbial biomass and necromass varied across the chronosequence; (3) whether microbial biomarkers were correlated with changes in SOC.

## 2. Materials and methods

### 2.1. Site description

Our study plots are located in the Changbai Mountain National Nature Reserve (42°20′–42°24′ N, 127°55′–128°06′ E, 780–920 m a.s.l.), which was established in Jilin Province (northeast China) in 1960. Mean annual temperature is 2.9 °C; mean annual precipitation is 700 mm, and mainly occurs between June and September. The slightly acidic soils (Supplementary Table 1) are classified as alfisols and formed through tephra and basalt weathering. The region is dominated by broad-leaved Korean pine mixed forests. Human disturbance (primarily logging) has created a reforestation chronosequence, with five dominant forest stand ages of approximately 20, 80, 120, 200 and ≥ 300 years (hereinafter referred to as ‘300 years’). These forests were allowed to regenerate naturally (i.e. without planting new trees).

Forest stands restored for 20 years are dominated by *Betula platyphylla* and *Populus davidiana*, two pioneer species used to define early succession. The middle successional forests (80 and 120 years) are dominated by *Acer mono*, *Fraxinus mandshurica*, *Quercus mongolica*, and *Tilia amurensis*. Late successional forests (200 and 300 years) are dominated by *Pinus koraiensis* (climax species), with contributions from *Acer mono*, *Fraxinus mandshurica*, *Quercus mongolica*, and *Tilia amurensis*. Shrub and herbaceous species colonizing the understory are similar across stand ages and include: *Vaccinium vitis-idaea*, *Lonicera edulis*,

*Philadelphus schrenkii*, *Corylus mandshurica*, *Sorbaria sorbifolia*, *Acanthopanax senticosus*, *Schisandra chinensis*, and *Syringa oblata*, among others.

### 2.2. Sampling and processing

We established study plots in August 2014 using a randomized within-forest nested design. At each of the five forest stand ages, we randomly selected six 15 × 20 m<sup>2</sup> plots, with a minimum spacing of 20 m between each plot. To capture spatial heterogeneity, we collected soil cores (0–15 cm) and pooled plant litter samples, collected near each soil core, from ten random subplots. Subplots were then aggregated to the plot level by compositing samples into one representative litter and one representative soil sample (n = 6 samples per forest stand age). All samples were stored in Ziploc bags and shipped to the laboratory at ~0 °C. Soils were homogenized, sieved to 2 mm, and subsampled for analyses as below.

### 2.3. Litter and soil physicochemical analyses

Total C and nitrogen (N) concentrations were measured on oven-dried litterfall (60 °C for 48 h) and air-dried soil. Litter and soil samples were ground, and total C and N concentrations were determined using a Vario MACRO Cube (Elementar Analysensysteme GmbH, Langenselbold, Germany). We measured pH using the soil slurry method, where air-dried soil was shaken in boiled water (soil: water [m/v] = 1:2.5) for 2 min, the slurry was allowed to settle for 30 min, and the supernatant pH was measured using an electrode. Due to the acidic nature of these soils, we defined total soil C as SOC. Soil bulk density (BD) was measured using the cutting-ring method and calculated by dividing the dry mass of each composite soil sample by the sample volume. We estimated SOC storage using the following formula: SOC storage (Mg C ha<sup>-1</sup>) = SOC (%) × BD (g cm<sup>-3</sup>) × sample depth (cm). SOM compounds (i.e. aromatic C groups and polysaccharides) were evaluated using mid-infrared spectroscopy (mid-IR) (experimental details provided in Supplemental files).

### 2.4. Lipid analysis

Lipids were extracted and analyzed according to a phospholipid fatty acid (PLFA) procedure described by Frostegård et al. (1993). Briefly, 2 g of each soil was lyophilized and extracted using a chloroform/methanol/phosphorus-buffer at a 1:2:0.8 ratio. After the organic phase was separated, purified, and subjected to methyl esterification, lipids were analyzed using an Agilent 7890B GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a HP-ULTRA 2 column (25 m × 0.20 mm × 0.33 μm) and flame ionization detector. We used MIDI software (“Sherlock Microbial Identification System”, MIDI Inc., Newark, DE, USA) to identify individual PLFA peaks. We converted peak areas to μg lipid biomass g<sup>-1</sup> soil by referencing an internal standard peak (19:0) with a known concentration. Microbial lipid biomass was calculated as the sum of all identified peaks (detectable at > 0.5% and C atoms numbers < 20). Microbial functional composition was binned by the biomarker PLFAs analyzed within this dataset as follows: gram-positive (Gm<sup>+</sup>) bacteria (14:0iso, 15:0iso, 15:0anteiso, 16:0iso, 17:0iso, and 17:0 anteiso); gram-negative (Gm<sup>-</sup>) bacteria (16:1ω7c, 17:1ω8c, 18:1ω5c, 18:1ω7c, 17:0cyclo, and 19:0cyclo); actinomycetes (10Me16:0, 10Me17:0, and 10Me18:0); arbuscular mycorrhizal fungi (AMF) (16:1ω5c); saprotrophic fungi (SF) (18:1ω9c and 18:2ω6c). Ectomycorrhizal fungi could not be independently and specifically assigned using this method.

### 2.5. Amino sugar analysis

Amino sugars, including glucosamine (GluN), galactosamine (GalN), and muramic acid (MurA), were analyzed according to the

protocol of Zhang and Amelung (1996). Briefly, air-dried soil samples containing > 0.3 mg N were sieved to 0.15 mm, and hydrolyzed with 10 ml 6M HCl at 105 °C for 8 h. The hydrolysate containing 100 µl myo-inositol (internal standard) was filtered through a standard filter paper with a nominal pore size of 30–50 µm. Each hydrolysis flask was rinsed using ~3 ml deionized water and filtrates were dried at 52 °C under vacuum on a rotary evaporator. Dried residues were re-dissolved with ~6 ml deionized water and purified by KOH neutralization to precipitate metal ions and other organic molecules. The supernatant was fully dried at 52 °C under vacuum using a rotary evaporator. Next, 5 ml absolute methanol was added to dissolve the residues, which were then transferred to a vial and dried by N<sub>2</sub> gas at 45 °C. The dried residue was re-dissolved using 1 ml deionized water and 100 µl standard N-methylglucamine, and lyophilized.

To prepare the aldononitrile derivatives, lyophilized residues containing amino sugars were dissolved in 300 µl derivatization reagent containing 32 mg ml<sup>-1</sup> hydroxylamine hydrochloride and 40 mg ml<sup>-1</sup> 4-dimethylamino-pyridine in pyridine-methanol (4:1 v/v). The solution was fully mixed by swirling and heating at 75–80 °C for 35 min. After cooling to room temperature, the derivatives were reheated with 1 ml acetic anhydride at 75–80 °C for 25 min. After acetylation, 1.5 ml dichloromethane and 1 ml 1M HCl were successively added. The mixture was vortexed at room temperature for 30 s to isolate the organic phase, which was washed three times with 1 ml deionized water to thoroughly remove residual anhydride. Then, the remaining organic phase was dried by N<sub>2</sub> gas at 45 °C. Finally, the amino sugar derivatives were re-dissolved with 200 µl ethyl acetate-hexane (1:1) and analyzed on an Agilent 7890B GC (Agilent Technologies, Santa Clara, CA, USA) equipped with a HP-5 column (30 m × 0.25 mm × 0.25 µm) and flame ionization detector. Amino sugar extracts (1 µl) were injected into the column using N<sub>2</sub> as the carrier gas at a constant flow rate of 0.6 ml min<sup>-1</sup>. The GC inlet was set to 250 °C and operated in split mode with a 30:1 ratio. The individual amino sugar derivatives were separated by referencing the retention times of authentic standards containing GluN, GalN, and MurA. We normalized amino sugar-C contents by their molecular mass and calculated total soil amino sugar-C as the sum of GluN-C, GalN-C and MurA-C using the following equation: GluN-C = 72\*GluN/179.2, GalN-C = 72\*GalN/179.2, and MurA-C = 108\*MurA/251.23. We calculated amino sugar-C storage (Mg C ha<sup>-1</sup>) as amino sugar-C (mg kg<sup>-1</sup>)/10<sup>5</sup> × BD (g cm<sup>-3</sup>) × sample depth (cm).

## 2.6. Statistical analysis

All statistical analyses were performed using SAS V.8.1 software (SAS Institute Inc., Cary, NC, USA). We tested for the main effects of reforestation on soil C and N concentrations, SOC storage, microbial lipids, and microbial amino sugars using one-way analysis of variance (ANOVA) with Tukey's *post-hoc* tests. We used generalized linear models (GLM) with Gaussian (i.e. normal) distribution to evaluate the relationships between SOC storage and microbial lipids, microbial amino sugars, SOC concentration, or BD. Quadratic regression equations were used to fit changes in each dependent variable of interest (e.g. SOC concentration, microbial lipid biomass, amino sugar-C concentration, the ratio of amino sugar-C to SOC) over the course of reforestation. We used logistic functions to fit changes in SOC storage, amino sugar-C storage and the ratio of amino sugar-C to microbial lipid biomass.

## 3. Results

### 3.1. Soil organic carbon dynamics during reforestation

We observed a significant effect of reforestation on SOC concentrations, which ranged from 139.09 g kg<sup>-1</sup> to 190.39 g kg<sup>-1</sup>. Forest SOC concentrations increased by 36.42% in stands that were restored

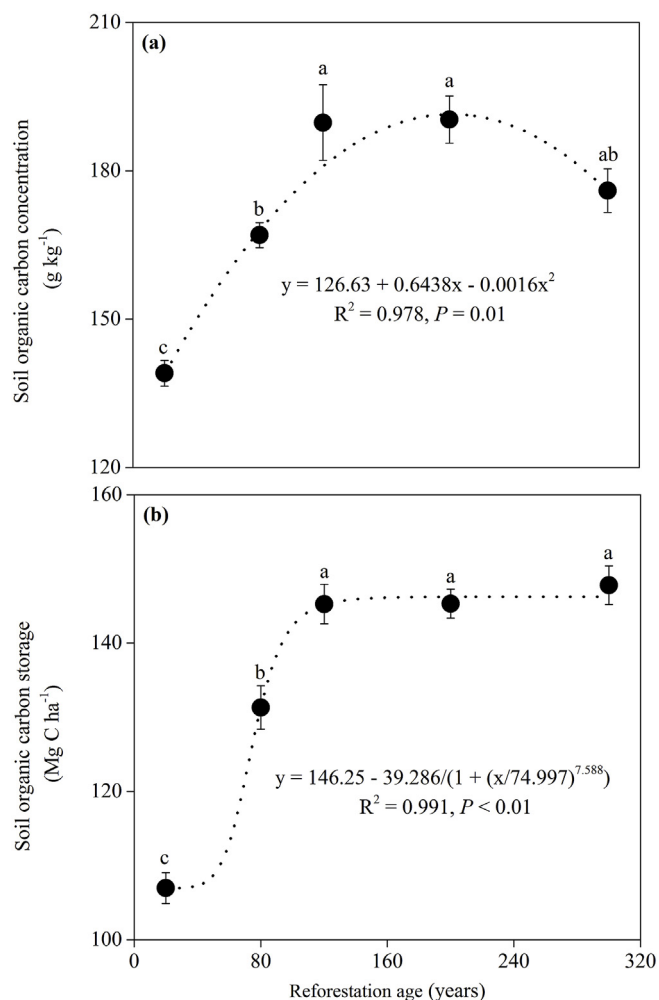
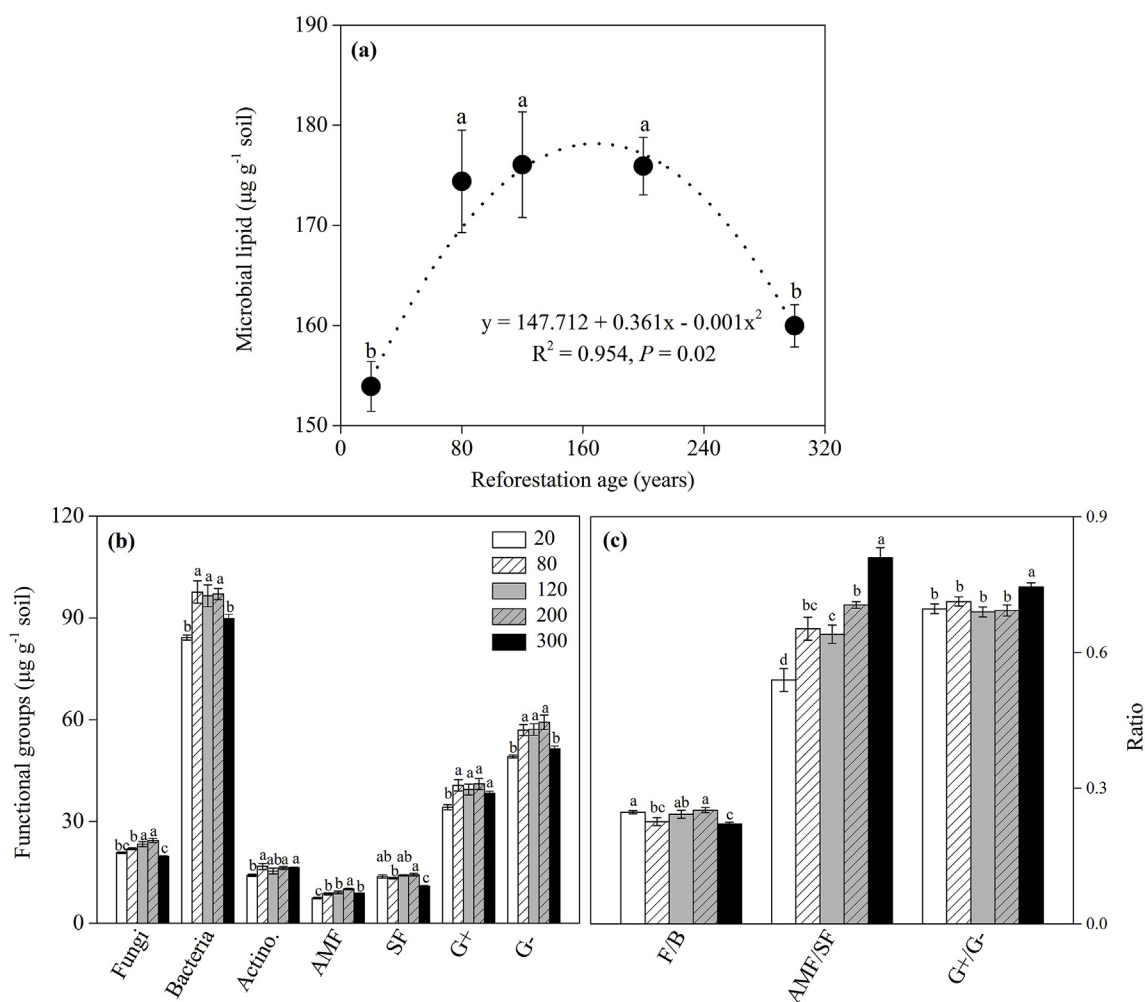


Fig. 1. SOC concentrations (panel a) and storage (panel b) dynamics during reforestation with average stand ages of 20, 80, 120, 200 and ≥ 300 years. SOC concentrations and SOC storage are estimated, respectively, as a quadratic and logistic function of time since reforestation. Symbols represent means ± SE (n = 6); different letters indicate significant differences at  $p < 0.05$ .

for 120 years relative to those that were restored for only 20 years ( $df = 29$ ,  $n = 30$ ;  $p < 0.001$ , Fig. 1a). The highest SOC concentrations were detected after 120 years and declined by 8.17% after 300 years of restoration ( $p > 0.05$ , Fig. 1a). SOC storage exhibited similar patterns, ranging from 107.0 Mg C ha<sup>-1</sup> (1 Mg = 10<sup>6</sup> g) to 147.8 Mg C ha<sup>-1</sup> ( $p < 0.001$ ). However, SOC storage did not decline between 120 and 300 years ( $p > 0.05$ ; Fig. 1b). The rate of SOC accumulation was 0.38 Mg C ha<sup>-1</sup> year<sup>-1</sup> from 20 to 120 years. Soil BD remained consistent across stands ranging in age from 20 to 200 years (average of 0.52 g cm<sup>-3</sup>;  $p > 0.05$ ), but increased by 8.91% in 300-year-old forests ( $p < 0.05$ ; Supplementary Table 1). The relative abundance of aromatic C functional groups gradually increased ( $p < 0.001$ ) while the relative abundance of polysaccharide functional groups gradually decreased ( $p < 0.001$ ) as reforestation advanced (Fig. S1).

### 3.2. Microbial lipids during reforestation

Soil microbial lipid concentrations (i.e. total PLFA biomass) significantly increased in soils underlying forests that had recovered between 20 and 200 years (from 153.90 µg g<sup>-1</sup> to 176.06 µg g<sup>-1</sup>) but declined by 9.98% in 300-year-old forests (159.97 µg g<sup>-1</sup>) to levels observed in recently disturbed soils (Fig. 2a). The composition and abundance of microbial functional groups, represented by specific lipid



**Fig. 2.** Changes in total microbial lipid biomass (panel a), microbial functional groups (panel b), and ratios of microbial functional groups (panel c) during reforestation. The dotted line represents a fitted quadratic function of total detectable microbial lipid biomass against time since reforestation. Actino., actinomycete; AMF, arbuscular mycorrhizal fungi; SF, saprotrophic fungi;  $\text{G}^+$ , gram-positive bacteria;  $\text{G}^-$ , gram-negative bacteria; F/B, ratio of fungi to bacteria. Symbols (panel a) and bars (panels b and c) represent means  $\pm$  SE ( $n = 6$ ); different letters indicate significant differences at  $p < 0.05$ .

biomarkers, were significantly influenced by reforestation (Fig. 2b). While fungal and bacterial abundance exhibited similar patterns across the reforestation chronosequence, bacterial abundance plateaued after 80 years, while fungal abundance did not plateau until 120 years. In soils underlying 300-year-old forests, both fungal and bacterial abundance dropped to levels observed in recently disturbed soils ( $p < 0.05$ ). The abundance of actinomycetes, AMF,  $\text{G}^+$  bacteria, and  $\text{G}^-$  bacteria all increased with forest age ( $p < 0.05$ ). While the abundance of  $\text{G}^+$  and  $\text{G}^-$  bacteria stabilized after approximately 80 years, AMF abundance continued increasing up to approximately 200 years (Fig. 2b). Across all microbial functional groups, we observed significantly different communities colonizing the restored forests (Fig. 2c), with significant increases in fungal to bacterial ratios from 80 years to 200 years ( $p < 0.05$ ), and gradual increases in AMF to SF fungal ratios from 20 years to 300 years of restoration ( $p < 0.001$ ).

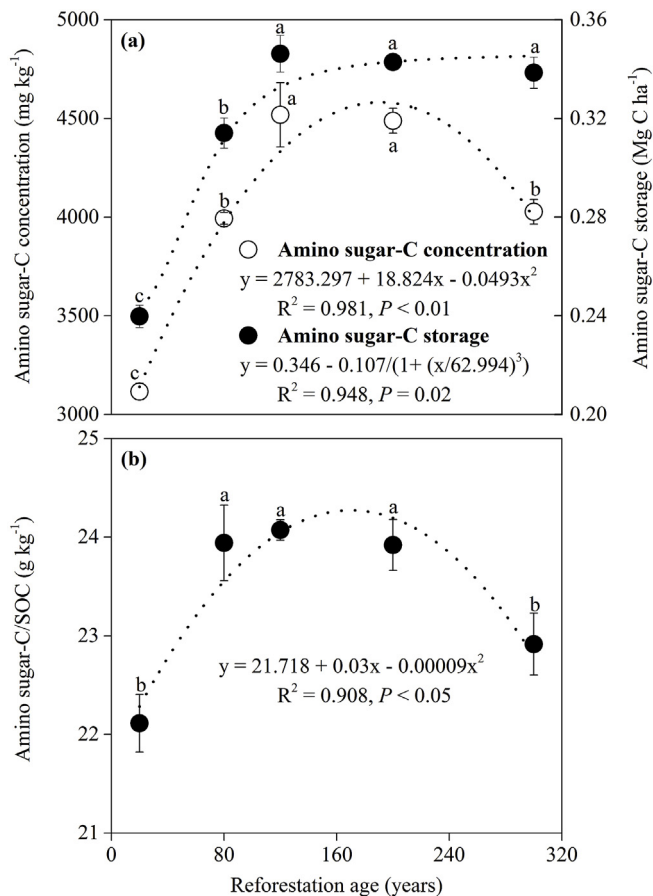
### 3.3. Microbial amino sugars during reforestation

Total amino sugar-C concentrations increased during the early stages of reforestation, stabilized after approximately 120 years, and declined significantly in the oldest forests (Fig. 3a; Supplementary Table 1). Amino sugar-C storage ranged from  $0.24 \text{ Mg C ha}^{-1}$  to  $0.35 \text{ Mg C ha}^{-1}$  and increased by 44.42% before stabilizing after approximately 120 years (Fig. 3a). In addition, the contribution of

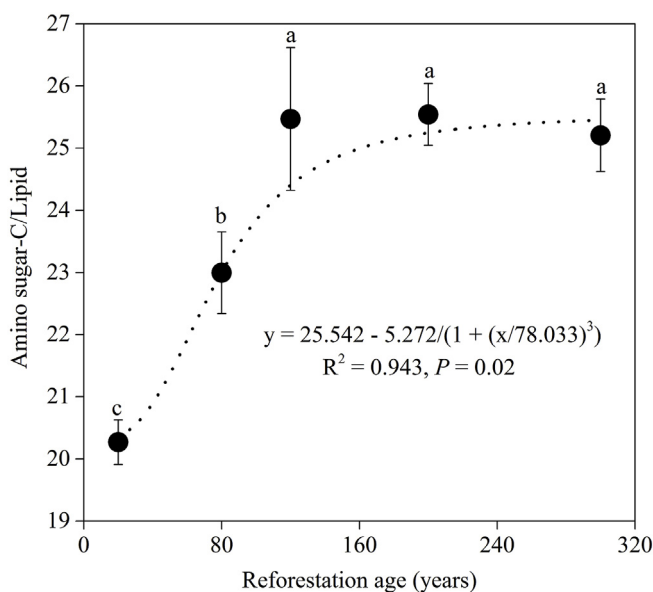
microbial necromass to belowground soil C (represented by the ratio of amino sugar-C to SOC) increased by 8.85% ( $p < 0.001$ ) during the early and middle stages of reforestation; the contribution in 300-year-old forests was similar to recently disturbed soils (Fig. 3b). Intriguingly, compared to the change in microbial biomass and necromass, the ratio of amino sugar-C to microbial lipid plateaued after approximately 120 years of restoration (Fig. 4), potentially reflecting a true balance between microbial production and death within the chronosequence.

### 3.4. Impacts of microbial lipids (biomass) and amino sugars (necromass) on SOC storage

We explored the relationships of SOC storage to microbial biomass and necromass to assess microbial mediation of, and contribution to, SOC accumulation along a reforestation gradient (Fig. 5). SOC storage was positively correlated with total microbial lipid ( $R^2 = 0.14$ ,  $p = 0.04$ ; Fig. 5a) and bacterial biomass ( $R^2 = 0.16$ ,  $p = 0.02$ ; Fig. 5c), but not with total fungal biomass (Fig. 5b). However, AMF, the dominant fungal functional group, was positively correlated with SOC storage ( $R^2 = 0.44$ ,  $p < 0.001$ ; Fig. 5e). The strong correlation between AMF/SF and SOC storage ( $R^2 = 0.45$ ,  $p < 0.001$ ; Fig. 5j) suggests a mechanistic link exists between SOM accumulation and microbial community composition. This link was further supported by the strong relationship between SOC storage and microbial necromass, as assessed



**Fig. 3.** Dynamics of soil amino sugar-C concentrations and storage and microbial C contributions during reforestation. Microbial C contributions are estimated as the ratio of amino sugar-C to SOC. Changes in amino sugar-C concentrations (panel a, open symbols) and microbial C contributions (panel b) are estimated as a quadratic function of time since reforestation. Changes in amino sugar-C storage (panel a, closed symbols) are estimated as a logistic function of time since reforestation. Symbols represent means  $\pm$  SE ( $n = 6$ ); different letters indicate significant differences at  $p < 0.05$ .



**Fig. 4.** The ratio of amino sugar-C to microbial lipid biomass as a logistic function of time since reforestation. Symbols represent means  $\pm$  SE ( $n = 6$ ); different letters indicate significant differences at  $p < 0.05$ .

by amino sugar-C concentrations ( $R^2 = 0.68$ ,  $p < 0.001$ ; Fig. 5i) and storage ( $R^2 = 0.88$ ,  $p < 0.001$ ; Fig. 5m). For reference, the relationships between SOC storage and SOC concentration or BD are also shown; SOC storage and SOC concentrations were positively correlated ( $R^2 = 0.70$ ,  $p < 0.001$ ; Fig. 5n), but there was no relationship between SOC storage and BD (Fig. 5o).

## 4. Discussion

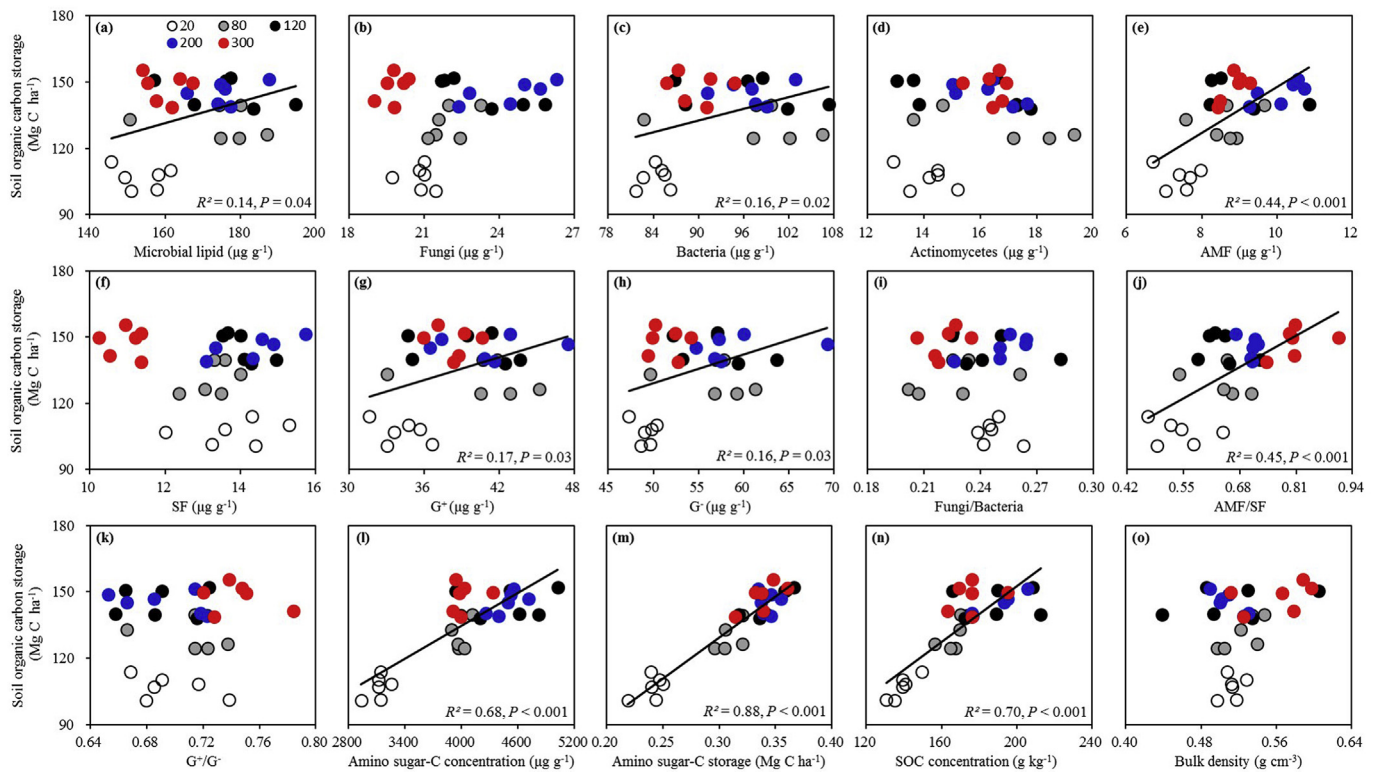
### 4.1. SOC accumulation during reforestation

In terrestrial ecosystems, the balance between C retention and release is influenced by ecosystem management and land use change, which determine whether soils are a sink or source of atmospheric C (Carvalhais et al., 2014; Laganier et al., 2010). We found that reforestation significantly increased SOC accumulation across the chronosequence, suggesting the implementation of sustainable forest-management practices could mitigate the effects of climate change. While the role of vegetation in determining soil C pool dynamics has been well established (Guo and Gifford, 2002; Ha et al., 2018), we suggest microbial communities play an equally critical role in sequestering C belowground.

The link between ecosystem restoration and the longer-term recovery of ecosystem structure and function suggests forest C sequestration capacities are driven by the systematic transformation of C through plant and microbial networks (Peichl and Arain, 2006; Anderson et al., 2017). SOC accumulation during forest restoration is tightly linked to the recovery of plant diversity and abundance (Jandl et al., 2007; Deng et al., 2013). Greater plant productivity can translate to belowground C storage through two pathways (1) greater inputs of plant residues, including leaf and root litter, or (2) enhanced input of photosynthetic C through root exudation of low molecular weight compounds. Relatively young forests, in early or middle successional stages, sequester more CO<sub>2</sub> in plant biomass and release more C belowground via plant litter and root exudates than forests in late succession (Xiao et al., 2017; Gao et al., 2018). In contrast, older and relatively N-limited systems may reduce microbial substrate use efficiencies and the retention of plant-derived C belowground. In the early and middle stages of succession, plant litter C to N ratios were relatively low, suggesting the inputs of N-rich plant matter facilitates SOC accumulation. Moreover, we found that leaf litter collected from old forest stands had significantly higher C to N ratios than litter collected from younger forests (Supplementary Table 1), which has been linked to reductions in photosynthetic capacity and plant productivity (Wright et al., 2005; Xiao et al., 2017). However, these results cannot explain why soil C storage remained relatively constant, and even increased slightly during the later stages of reforestation (e.g. 200 years and 300 years). In addition to plant effects on SOC accumulation during reforestation, we speculate the accumulation of soil C is driven by additional biotic factors, specifically changes in microbial community composition and function that structure the link between plant and soil C (Schimel and Schaeffer, 2012; Wieder et al., 2013).

### 4.2. Microbial mediation in SOC accumulation

The relationship between SOC storage and microbial lipid concentrations during reforestation suggests microbial communities facilitate SOM formation and retention belowground. Microorganisms play a critical role in maintaining ecosystem productivity by providing nutrients (e.g. nitrogen) to plants via SOM decomposition and turnover (Zak et al., 2003; McGuire and Treseder, 2010). The positive relationship between microbial biomass and reforestation suggests microbial communities liberate enough N (e.g. NH<sub>4</sub><sup>+</sup>-N and NO<sub>3</sub><sup>-</sup>-N) to meet plant demands (Supplementary Table 1). In this process, stoichiometrically balanced plant growth sequesters C in biomass and increases litter and root exudate inputs belowground, increasing the deposition of



**Fig. 5.** Linear regression of SOC storage against microbial lipid parameters (a–k), amino sugar parameters (l–m), SOC concentrations (n), and bulk density (o) across all stages of reforestation (n = 30). The five average forest stand ages are identified by different symbols as follows: 20 years (open circles), 80 years (grey circles), 120 years (black circles), 200 years (blue circles), 300 years (red circles). Solid lines depict significant linear relationships between SOC storage and potential predictor variables ( $p < 0.05$ ), while plots without regression lines are not linearly related ( $p > 0.05$ ). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

#### C belowground as forests age.

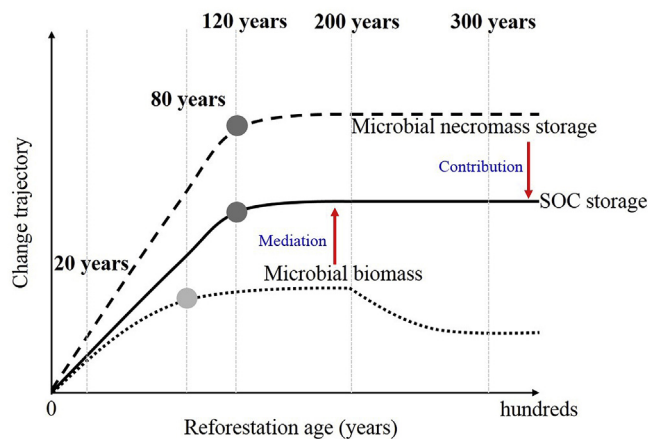
In older forests (i.e. 300 years), SOC concentrations declined slightly while total SOC storage remained relatively constant, likely resulting from higher soil bulk densities. In contrast, microbial biomass declined substantially relative to SOC concentrations. These patterns are consistent with other findings (Tang et al., 2009; Blažko et al., 2015) and may be linked to changes in the abundance or composition of the microbial community and their carbon use efficiencies (CUE). Our Mid-IR analysis revealed a gradual increase in the relative abundance of aromatic C groups with forest age (Fig. S1, Shao et al., 2019). Aromatic C functional groups (attributed to lignin) are considered unfavorable substrates for microbial assimilation (Fuchs et al., 2011; Suseela et al., 2013) and may explain reductions in microbial CUE as forests age (Fig. S2, where CUE is estimated as the ratio of microbial lipid to SOC concentration (Sparling, 1992; Wang et al., 2009)). Alternatively, the inconsistent changes between microbial biomass and SOC concentrations in older forests could be explained by lower overall microbial activity, reduced catabolic potential, and shifts in microbial community composition.

Forest restoration alters microbial community structure by changing the quantity and chemical composition of plant inputs (Bonfante and Anca, 2009; Pausch et al., 2016). We found that bacterial communities proliferated during the earliest stages of forest succession, reaching peak biomass before fungal communities. During early successional stages, forests were dominated by *Betula platyphylla* and *Populus dayidiana*. While low vascular plant diversity and recent soil disturbance have been shown to limit the development of robust mycorrhizal networks (Neuenkamp et al., 2018), they have less pronounced effects on bacterial diversity. These findings are consistent with previous work (Zhou et al., 2017) that suggest bacteria outcompete fungi when plant biomass and diversity is low. During this period, bacteria may play an important role in soil C cycling by decomposing SOM to supply

nutrients that promote subsequent plant growth and recruitment. Because many fungal communities establish symbiotic relationships with plants (Bonfante and Anca, 2009; Brundrett and Tedersoo, 2018), they are more sensitive to changes in vegetation and land-use change than bacteria. Regrowth of diverse plant communities during forest restoration may therefore facilitate fungal relative to bacterial dominance, which is consistent with our findings and others (Zak et al., 2003; Bardgett et al., 2007; Tarlera et al., 2008). We also observed a strong relationship between soil C storage and AMF biomass, suggesting symbiotic fungi are critical mediators of SOC storage (Clemmensen et al., 2013). While we could not directly evaluate the contribution of ectomycorrhizal fungi (ECM) to total fungal biomass using PLFA biomarkers, several studies within the same forest found the contribution of ECM to total microbial biomass was relatively minor, which they attributed to relatively high N availability and dominance of AMF and SF communities (He et al., 2017; Yao et al., 2017). Together, our findings highlight mechanisms by which microbial biomass and community composition drive SOC cycling and storage during reforestation.

#### 4.3. Microbial contribution to SOC accumulation

Although microbial communities regulate soil processes and functions (e.g. C cycling), living microbes account for only 1–5% of total SOC (Dalal, 1998). However, the rate of microbial turnover is rapid, suggesting necromass is a critical pathway of SOC formation and reflects temporal ecosystem dynamics (Glaser et al., 2004; Kallenbach et al., 2016). We found that microbial necromass increased significantly during reforestation and may represent a direct pathway of SOC accumulation in young and middle-aged forests. Greater retention of amino sugars during reforestation may be caused by active assimilation of plant-derived C into microbial biomass, which eventually stabilizes through *in vivo* turnover pathways and preferential retention of



**Fig. 6.** Microorganisms are dynamically involved in SOC accumulation during reforestation. Different curves depict shifts in microbial parameters (i.e. microbial biomass and necromass) and SOC storage during long-term reforestation. Microbial biomass (dotted line) is an early and sensitive indicator of ecosystem recovery and represents the community actively mediating SOC storage (solid line). Microbial necromass (dashed line) contributes to the formation and retention of stabilized SOC pools. Microbial biomass peaks at approximately 80 years (light grey node), while SOC and microbial necromass do not peak until after approximately 120 years (dark grey nodes).

anabolic byproducts belowground (Liang et al., 2017).

We observed a decline in the contribution of microbial necromass to SOC stocks at the latest stage of reforestation (i.e. 300 years). The higher C to N ratio of plant litter from old growth forests can limit rates of decomposition and may have reduced the synthesis of microbial biomass and accumulation of microbial necromass. Additionally, a temperate forest landscape model simulating changes in plant biomass and diversity during 300 years of reforestation suggests plant mortality rates begin outpacing regrowth after ~200 years (Xiao et al., 2017). Reductions in root exudate deposition into the rhizosphere could reduce microbial biomass and help explain the relatively low amino sugar–N concentrations we observed in soils collected from old growth forests (Supplementary Table 1). Consistent changes in amino sugar–C with SOC suggest microbial necromass is a mechanistic indicator of SOC formation and storage (Lauer et al., 2011; Roth et al., 2011).

#### 4.4. Microbial parameters as indicators of SOC dynamics

To our knowledge, this is the first study that directly associates SOC cycling and formation with successional patterns in microbial community composition and necromass (Fig. 6). We found that microbial biomass pools stabilized earlier than SOC storage and responded to changing soil nutrient availability during forest recovery. These patterns suggest microbial communities both facilitate and respond to ecosystem recovery (Harris, 2009). Specifically, we suggest active microbial communities promote SOC accrual during the early and middle stages of reforestation, but respond to changes in plant diversity and litter quality as forests mature. As a result, microbial communities appear to be sensitive indicators of environmental change that rapidly adapt to and shape their environment. In contrast, slower turnover rates of microbial necromass can explain increases in SOC storage with long-term ecosystem development (Lauer et al., 2011). Amino sugars can therefore serve as an indicator of the ‘chronic’ responses that drive SOC accumulation.

The ratio of amino sugars to microbial lipids showed a consistent trend with SOC storage, with steady-state conditions establishing after 120 years of reforestation. This synchronicity suggests the ratio of microbial necromass to active biomass reflects an intrinsic balance between the presence and death of soil microbial members. As a result, we propose the ratio between necromass and biomass captures the

dynamic role that microbial communities play in mediating SOC formation and turnover better than either parameter alone. By explicitly representing the continuum between microbial death and growth, this index illuminates microbial contributions to soil C cycling within a developing ecosystem. Additionally, while microbial lipids reflect the current status of the microbial community (Frostegård et al., 2011), microbial residues reflect the legacy impacts of microbial metabolism (Amelung, 2001) on longer-term SOC storage (Liang et al., 2016) in forest soils. Thus, it is critical that future research describes microbe–soil C sequestration as a function of both microbial living biomass and necromass.

#### 4.5. Applications

Reforestation is widespread in many countries and projected to increase as a function of global demand (Crouzeilles et al., 2016; Houghton and Nassikas, 2018). Sustainable forest management practices improve forest ecosystem functions and services (Birdsey and Pan, 2015) and are an effective way of mitigating global climate change by sequestering C in plant biomass and soils (Cameron et al., 2017). As many ecological chronosequences do not have independent replicates, inferences are drawn from pseudo-replicated designs. However, global meta-analyses (covering > 200 forested sites) found that ‘time since reforestation’, rather than the geographical location of a particular forest, is the dominant factor influencing soil C stocks, biodiversity, and the degree of ecosystem recovery (Li et al., 2012; Crouzeilles et al., 2016). As a result, we believe our work has broad implications for reforestation, but further work is needed to rigorously test whether the ratio of microbial amino sugars to lipids can be used to evaluate the degree of forest recovery. Clarifying how microbial structure and function evolves throughout ecosystem remediation is essential in understanding how microbes mediate soil C cycling, and for predicting how forest ecosystems will respond to climate change. Together, our findings suggest global ecosystem restoration projects are beneficial for global health and sustainability (i.e. by facilitating soil carbon storage and re-establishing diverse plant and microbial communities), and represent an important tool for managing ecosystem C.

#### 5. Conclusions

Our results suggest reforestation serves as a long-term approach to offset CO<sub>2</sub> emissions resulting from deforestation, especially as C accumulation in soils underlying boreal forests can persist for centuries. We found that plant litter and other easily degradable SOM pools were converted into more persistent SOC through the “soil microbial pump” (Liang et al., 2017). This microbe-derived C contributed to SOC stability, enhancing the capacity of soils to mitigate environmental changes. Intriguingly, the ratio of necromass to active biomass appears to reflect a true biologic balance between production and death of the soil microbial members and may be a useful index for evaluating ecosystem recovery. We suggest explicitly including microbial dynamics in earth ecosystem models will enhance their ability to forecast global C dynamics, particularly in regions with shifting management and climate regimes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.soilbio.2019.01.012>.

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